Conditional deletion of Dicer from a subset of hippocampal neurons results in cell-autonomous changes in dendritic morphology

An Honors Research Distinction Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction in Neuroscience in the College of Arts and Sciences of the Ohio State University

By

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April, 2014

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Special thanks to Katelin Hansen for her guidance through the completion of this project

Abstract

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules involved in the post-transcriptional repression of target mRNAs. The gene silencing activity of miRNAs is important for a range of cellular processes including cell differentiation, proliferation, and cell cycle regulation. As part of a complex set of processing steps, a pre-miRNA hairpin structure is processed by an enzyme called Dicer into the mature miRNA. As, such, Dicer has been targeted using gene disruption approaches to gain insight into the functional role of miRNAs. Using these approaches, work to date in the central nervous system has shown that Dicer knockout mice exhibit disrupted neuronal maturation, and even neuronal death. Although these findings provide important information regarding the role of miRNA in the developing nervous system, we still know relatively little about the function of miRNA in the mature CNS. To begin to address this question, we employed a tamoxifen-inducible cre-lox system to selectively delete Dicer from forebrain neurons of adult mice. To further refine this approach, the expression of tamoxifen-inducible CRE recombinase was controlled by the Thy1 promoter. This approach allowed for the selective elimination of Dicer from only a handful of forebrain neurons, thus allowing us to examine the effect of miRNA in a cell-autonomous manner. In these studies, we found that the elimination of miRNA expression decreased neuronal spine density (spines are the locus for synapse formation) and altered neuronal dendritic branching, compared to control mice. By selectively deleting Dicer within the mature brain, we demonstrate that the miRNA class of RNA plays an important role in the regulation of neuronal structure, and, by implication, neuronal function. Given that the structure of a neuron mediates its ability to communicate with other cells and that memory and information processing in the mature nervous system are

dependent on activation within a distributed neural network, miRNAs may play an important role in normal cognitive activity.

Introduction

MicroRNA (miRNA) regulate protein synthesis through inhibition of messenger RNA translation in a time and tissue-specific manner (Lagos-Quintana et al., 2002; Kim et al., 2004). These evolutionarily conserved small non-coding RNAs bind to the 3' untranslated region (3' UTR) of target mRNAs via complementary base pairing, leading to sequestration or degradation of the target (Reviewed by Pillai, 2005). Cell death, patterning of the nervous system, and developmental timing have all been associated with miRNA regulation (Reviewed by Ambros, 2004), making them the focus of much investigation concerning normal and aberrant brain function.

miRNA are transcribed from intronic regions of the genome by RNA polymerase II and cleaved by Drosha to a ~70 nucleotide (nt) stem loop intermediate (pre-miRNA) in the nucleus. pre-miRNA are then transported by Ran-GTP and the export receptor Exportin-5 to the cytoplasm where mature miRNA are generated through cleavage of pre-miRNA hairpin structures by a dsRNA-specific RNase called Dicer. A single miRNA strand is then loaded into the RNA-induced silencing complex (RISC) where it interacts with members of the Argonaut protein family and is prepared for post-transcriptional repression of mRNAs (Reviewed by Bartel, 2004). Targeted knockout of the Dicer gene has been shown to prevent the formation of mature miRNA, resulting in abnormal brain morphogenesis in zebrafish (Giraldez, 2005), and is lethal in mice at the embryonic stage (Berstein, 2003), suggesting that the gene-silencing action

of miRNAs is essential for vertebrate development. While progress has been made in our understanding of miRNAs' influence in development, further investigation is needed into the role of Dicer in the mature CNS. Studies using α-calmodulin kinase II (αCaMKII) Cre to selectively ablate Dicer activity in excitatory forebrain neurons report that it is important for axonal pathfinding, apoptosis,and hippocampus-dependent spatial memory (Davis, 2008; Konopka 2010). Dicer deletion in embryonic dopaminergic cells leads to behavioral defects, decreased lifespan, and reduced brain size and mass (Cuellar 2008). Disregulation of Dicer expression has also been implicated in human conditions such as aging, diabetes, and schizophrenia (Yanqing et al., 2013; Beveridge et al., 2010). Approaches relying on extensive inactivation of Dicer throughout the forebrain limit our ability to determine whether observed changes are due to miRNA loss in individual cells or to a systemic response to cell death and altered brain morphology.

Here, we use a tamoxifen-inducible cre-lox system driven by the mouse thymus cell antigen-1 (Thy-1) promoter to selectively delete Dicer in a subset of forebrain neurons of adult mice (Deisseroth et al., 2006; Feil et al., 2009; Feng, 2000). This approach allows us to maintain a healthy extracelluar environment such that any neuronal changes are due to genetic deletion of Dicer within that cell, rather than as a result of surrounding pathological context. Our results indicate that inactivation of Dicer in these excitatory cells affects spinogenesis and dendritic morphology without causing significant cell death, further demonstrating the importance of miRNA in neuronal structural regulation.

Materials and Methods

Thy1-YFP;CreER$^{+/+}$ x Dicer$^{f/f}$ transgenic mice

To achieve tamoxifen-inducible, tissue-specific inactivation of Dicer, animals expressing CreER recombinase and yellow fluorescent protein (YFP) under the Thy1 promoter (Jackson Labs, strain 012708) were crossed to a line with the second RNase III domain of the Dicer gene flanked with loxP sites (Jackson Labs, strain 006366). These Thy1-YFP;CreER$^{+/+}$ x Dicer$^{f/f}$ transgenic mice were maintained in the absence of tamoxifen until 6 to 8 weeks of age. Recombinase activity was then induced using an IP injection of 100 mL of tamoxifen (20mg/mL) in corn oil solution once every 24 hours for 7 consecutive days. Control animals received IP vehicle injections of corn oil only. All animal breeding and experimental procedures were approved by the Ohio State University Animal Care and Use Committee (protocol number: 2008A0227).

PCR

The presence of the Thy1-YFP;CreER$^{+/+}$ and the floxed Dicer alleles were confirmed by PCR-based genotyping using GenScript Taq DNA Polymerase. The PCR reactions were then run in a 1% agarose gel and visualized using ethidium bromide.

Tissue processing and Immunofluorescence

Two weeks after tamoxifen-induced Cre recombination, experimental mice and age-matched controls were anesthetized using a ketamine/xylazine solution. Brain tissue was post-fixed via pericardial perfusion with 4% DEPC paraformaldehyde at 4ºC and 500mm-thick coronal sections were cryoprotected overnight in 30% sucrose in PBS. Hippocampal containing sections were sliced to 40 μm with a microtome and stored in a DEPC Ethylene Glycol solution. Tissue was prepared for immunofluorescent labeling by washing in PBS for a total of 15 min and

1% Triton X-100 in PBS for 5min. After a 1h blocking period in 10% normal goat serum (NGS) in PBS (room temperature), sections were incubated overnight at 4ºC in chicken anti-GFP antibody (1:2000) or rabbit anti-GFP (1:2500 acquired from Dr. Luc G. Berthiaume, University of Alberta, Canada) in 5% NGS. Following primary antibody labeling, sections were incubated (2h; room temperature; 5% NGS) in Alexa Fluor-488-conjugated goat anti-chicken IgG antibody (1:2000; Invitrogen, Carlsbad CA) or Alexa Fluor-594-cojugated goat anti-rabbit IgG antibody (1:500; Invitrogen). After each labeling step sections were washed in PBST (3x, 5 min per wash). Immuno labeled sections were mounted on slides with Fluoro-Gel (Electron Microscopy Sciences, Hatfield, PA).

Fluoro-Jade B Staining

Tissue labeled for YFP expression was mounted on sub slides in PBS and dried overnight before undergoing the following Fluoro-Jade B (FJB) procedures: 5 min of PBS, 1 min 95% EtOH to dehydrate the tissue, 1 min 70% EtOH to rehydrate the tissue, 2 min wash with water (3 times), 10 min wash with potassium permanganate (0.06% in water)) to reduce background brightness, 2 min wash with water (3 times), and 20 min in FJB (0.001% in 0.1% acetic acid). After drying the slides were washed with Xylene and mounted in DPX.

Morphological Analysis

Using tissue immunolabeled for YFP expression, images for the analysis of dendritic spines were captured at 63x with a 4.3x optical zoom on a Zeiss 510 confocal microscope and LSM Software Zen. Spine density was determined by quantifying the number of spines over a 10 μm dendritic segment 90-120μm from the cell soma.

Images for the analysis of CA1 basal dendrites were captured at 40x magnification using a 16 bit digital camera (Micromax YHS 1300; Princeton Instruments) mounted on an inverted Leica microscope (DM IRB). The number of primary basolateral dendrites was recorded along with the width of the apical dendrite at 20-30μm from the cell soma. The number of Thy1-YFP cells in the CA1 was recorded and averaged over two sections to obtain the mean number of cells per hemisphere.

Fluorescent *in situ* hybridization

Fluorescent *in situ* hybridization (FISH) against miRNA was carried out according to procedures outlined by Nuovo (2010). After undergoing immunofluorecent staining again Thy1-YFP (detailed above) tissue was mounted onto slides. Following a short digestion with pepsin, tissue was hybridized overnight with fluorescein-conjugated locked nucleic acid (LNA) probes (Obernosterer et al., 2007) to mouse miR-132 (Exiqon). The signal from the fluorescein-conjugated probes was amplified with an anti-fluorescein Alexa 488 signal detection kit (Invitrogen).

Statistical Analysis

The values in the paper are each given as a mean with standard error bars. Comparisons between groups were made using a Student's t-test with p,0.05 as the accepted significance level.

Results

To explore the role of miRNA in the mature CNS, we disrupted miRNA processing in a subset of cells through inducible conditional deletion of a functional region of Dicer. Using the Thy-1 promoter to drive the expression of tamoxifen-inducible Cre recombinase (Deisseroth et al.,

2006) we deleted the fourth exon encoding the second RNase III domain of the Dicer gene (Harfe et al., 2005) under temporally-controlled administration of tamoxifen in adult mice (Fig. 1a, b). These Thy-1 positive cells can be found throughout the hippocampus and specifically in the CA1. Cells expressing the transgene can be identified by the presence of yellow fluorescent protein (Fig. 1c). We confirmed that inactivation Dicer resulted in a marked absence of mature miRNA in CA1 pyramidal neurons positive for the Thy1-YFP marker (Fig. 1d). Thus, we proceeded to determine the resulting effect on cell death, spinogenesis, and dendritic morphology on a cell-autonomous level.

Given that disruption of Dicer functionality results in a complete loss of mature miRNA, we may expect death of affected Thy1-YFP positive cells. Using Fluoro-Jade B as a marker for degenerating neurons, we found that there was little to no cell death in control hippocampi or those treated with tamoxifen (Fig. 2). This suggests that Dicer deletion, and the subsequently altered levels of miRNA, has an insignificant role in the process of cell death in mature neurons. Therefore, any changes observed in neuronal morphology or connectivity were due to the induced reduction of mature miRNA and not a response to apoptosis in neighboring cells or brain structures.

Dendritic spines, as the sites of synaptic transmission between neurons, are essential to cell communication and thus important when studying cell structure. Using immunofluorescence to visualize Thy1-YFP;CreER$^{+/+}$ cells, a reduction in spine density was observed in basolateral dendrites of CA1 pyramidal neurons lacking functional Dicer (Fig. 3). Therefore, Dicer effects dendritic spine density and has a role in synaptic transmission on a morphological level.

To further study possible structural differences in Dicer deficient cells, we compared immunolabeled tissue from control animals and tamoxifen-treated litter mates and found a significant decrease in the number of primary basolateral dendrites in the experimental group (Fig. 4a, b). There was an observed trend toward a decrease in apical dendrite thickness in induced animals compared to controls but this reduction in size was not statistically significant (Fig. 4c). Another interesting finding was that the average number of Thy1-YPP positive neurons per hemisphere was significantly greater in cells lacking Dicer (Fig. 4d, e). These findings show that Dicer is involved in neuronal morphology in mature hippocampal cells.

Discussion

Here, we demonstrate that functional deletion of Dicer in a subset of excitatory forebrain neurons in adult mice leads not only to the downregulation of mature miRNA, but also results in abnormal dendritic structure. The use of tamoxifen-inducible Cre recombinase to inactivate Dicer allowed us to eliminate the influence of widespread cell death and toxicity on the system, highlighting the cell-autonomous effects of impaired miRNA functionality. We focused our observations on neuronal morphology, specifically changes in dendrites and their spines. Using miRNA-132 expression as validation, we showed that removal of Dicer leads to an overall reduction in the levels of mature miRNA, confirming that Cre recombination was successful in these cells (Bernstein et al., 2003).

The impairment of miRNA processing through Dicer deletion has previously been shown to result in neurodegeneration and to affect cell survival (Kim et al., 2007; Shaefer et al., 2007; Hebert et al., 2010). However, studies in which Dicer was deleted from excitatory forebrain

neurons *in utero* report an increase in cell death at postnatal day 0 but not at P15 (Davis, 2008). Hence we examined the possibility of increased cell death in the Thy1-YFP positive cells treated with tamoxifen. The lack of FJB-positive cells indicates that Dicer function, while essential for neuronal survival and morphogenesis in the developing brain, may not be as central to the maintenance of cell populations in postnatal forebrain structures (Hong et al., 2013, Cuellar et al., 2008, Konopka et al., 2010).

Spine density and structural abnormalities in synapses have been implicated in a number of neurological diseases, such as schizophrenia, Alzheimer's, and autism spectrum disorders (Garey et al., 1998; Penzes et al., 2011), and thus, the role of miRNA regulation in the production and maintenance of dendritic spines is potentially of interest for a range of etiologies. Dendritic spine length along with the total number of immature filopodia-shaped spines is increased when there is a removal of functional miRNA (Davis et al., 2008, Konopka et al., 2010). It is known that neuronally-enriched miRNA-134 acts as a negative regulator of spine volume and width in the hippocampus (Schratt et al., 2006). Moreover, in cultured hippocampal neurons miR-132 expression increases spinogenesis (Wayman et al., 2008) and *in vivo* overexpression of miR-132 also leads to an increase in spine density (Hansen et al., 2010). These studies suggest a prominent role for miRNA in the structural regulation of dendritic spines. Indeed, here we observed a marked decrease in spine density for neurons in which Dicer had been removed by Cre recombination.

Morphological changes in Dicer-ablated cells extended beyond the level of spine density to include overall remodeling of dendritic structure. The observed decrease in primary basolateral dendrites in miRNA-deficient cells is consistent with previous findings that the

absence of Dicer influences apical and dendritic branching and delays neurite outgrowth in cultured mature neurons (Davis et al., 2008; Hong et al., 2013). miRNA implicated in spinogenesis have also been found to influence neurite development (Vo et al., 2005) and the absence of such miRNA could account for the difference in dendritic morphology in these cells.

In addition to morphological changes in Dicer-deleted cells, we also observed an overall increase in the number of Thy1-YFP cells in tamoxifen-treated hippocamppi. While this observation is initially counter-intuitive, the reduction of dendritic spines and branching could lead to impaired connectivity resulting in compensation through increased cell survival. When Dicer is removed from postmitotic neurons of the mouse cerebral cortex there is a reported increase in neuronal cell packing density without a significant change in the number of cells (Hong et al., 2013). Since neuronal cell volume and not neuron number has previously been used to explain the changes in distribution of Dicer KO cells, further investigation is needed into the mechanism of cell survival in Thy1-YFP cells after inhibition of miRNA biogenesis.

Our study adds to the growing body of knowledge concerning the role of Dicer and mature miRNA in the structural connectivity between neurons. Future examination of the functionality of the dendritic spines in Dicer ablated cells as well as the electrophysiological activity of the neurons could provide more insight into miRNA regulated dendritic structure.

Figures

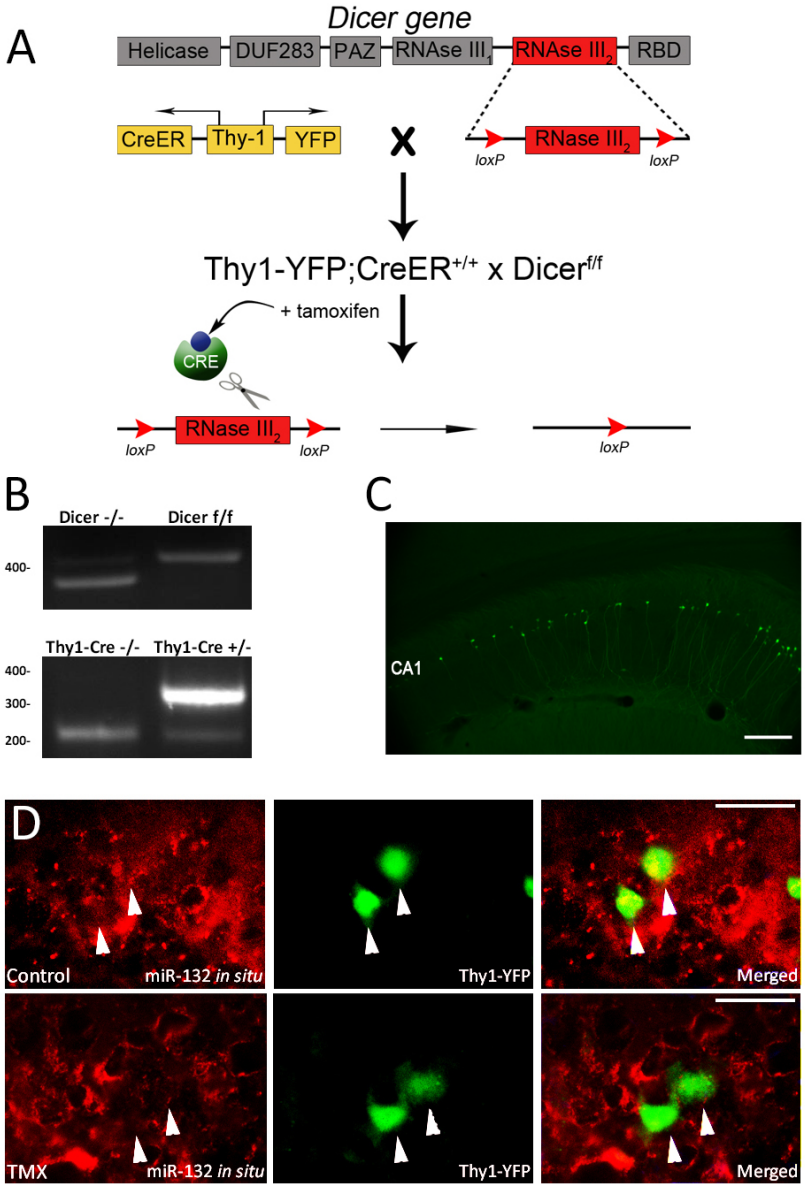


Figure 1. Conditional knock out of Dicer results in absence of mature miRNA. (A) Schematic of the Dicer targeting construct. The second RNase III domain of Dicer was floxed with loxP sites. Cre recombinase activity was induced by 7 consecutive days of tamoxifen treatment to inactivate Dicer. (B) PCR-based genotyping results confirming the presence of the Thy1-CreER and Dicer$^{f/f}$ genes. All animals used for experiments were homozygous for both genes. PCR reactions were run in a 1% agarose gel and visualized using ethidium bromide. (C) Thy1-YFP positive cells are found throughout the CA1 of the hippocampus. This immunofluorescent labeling technique was used for the study of dendritic morphology in these cells. Scale bar = 100 µm. (D) Fluorescent *in situ* hybridization (FISH) using an LNA anti-sense probe directed against miR-132 demonstrates the absence of mature miRNA in Thy1-YFP positive cells (white arrows) after tamoxifen induced Cre recombination. Scale bar = 90 µm.

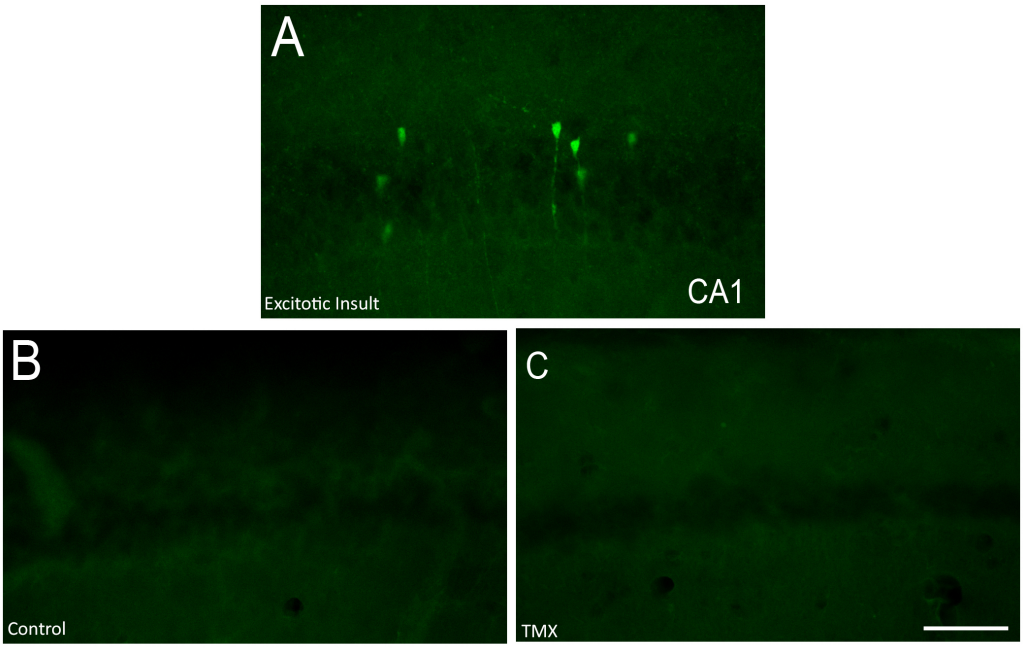


Figure 2. No observed cell death in Dicer ablated cells. Fluoro-Jade B staining for degenerating neurons shows no apparent cell death in cells maintaining functional Dicer and those with Dicer ablated. Tissue from mice receiving a pilocarpine injection was used as a positive control for excitotic insult (A) for comparison with control animals (B) and tamoxifen treated tissue (C). Scale bar = 50μm.

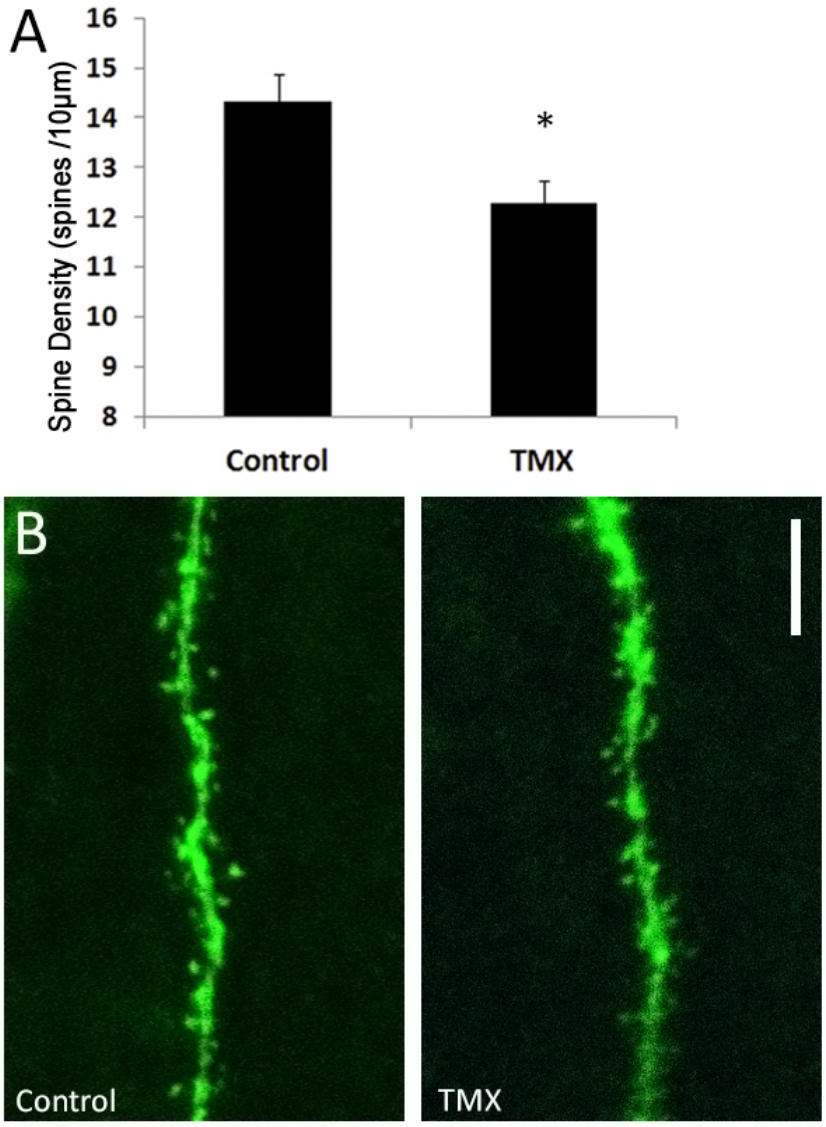


Figure 3. Disrupting miRNA biogenesis affects dendritic spine density. (A) Reduced spine density in Dicer ablated cells compared to genetically matched controls. Please see the Methods section for a description of the quantification methods. (B) Representative confocal images of CA1 pyramidal neuron basal dendrites in Thy1-YFP;CreER$^{+/+}$ cells maintaining Dicer levels and with Dicer inactivated by Cre recombination. Data are presented as mean spines per 10 µm ± SEM. *$P < 0.05$, two-tailed t-test, n = 10 animals per condition. Scale bar: 5 µm.

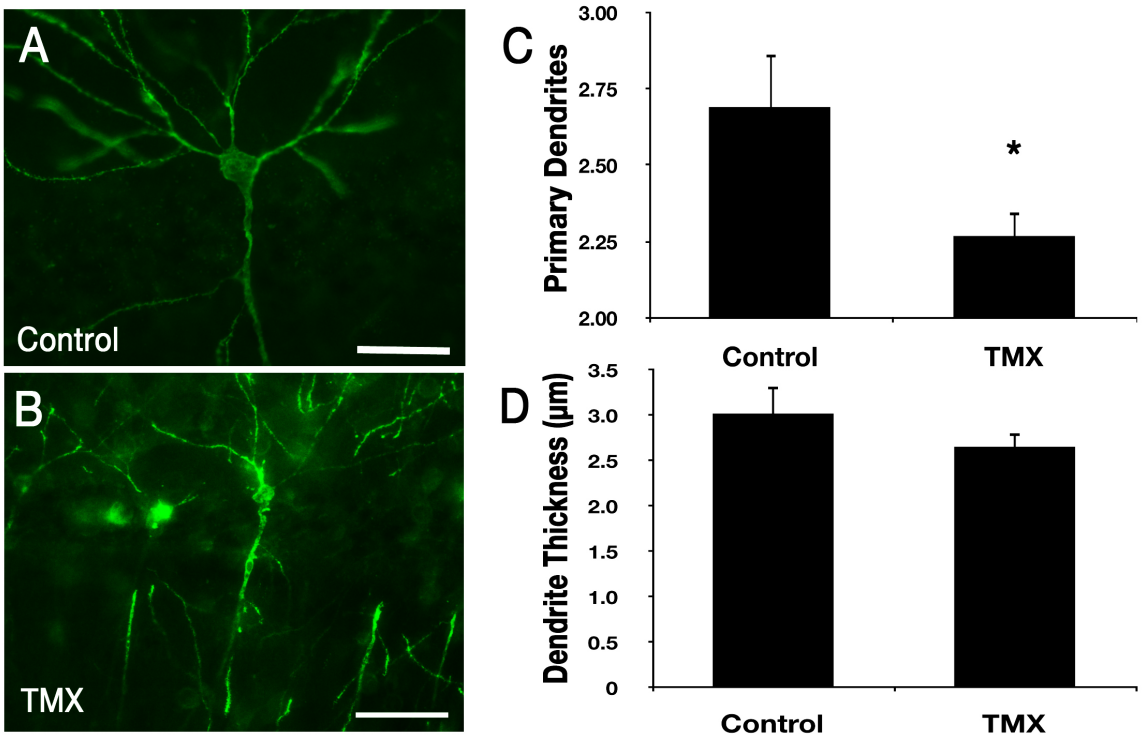
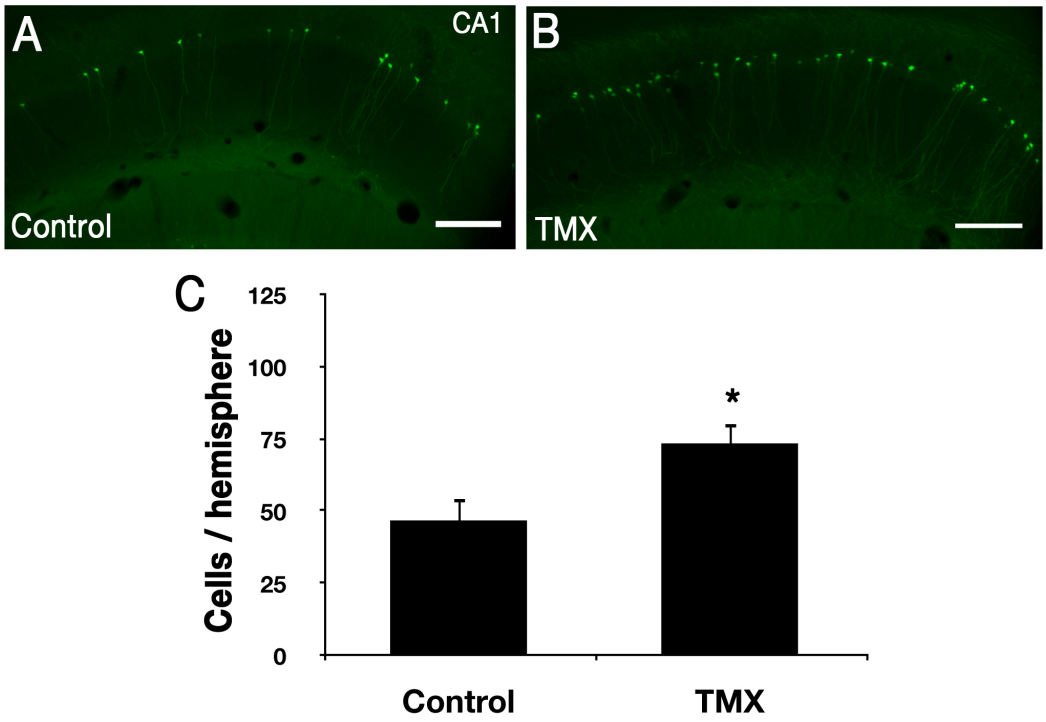


Figure 4. Morphological changes in Dicer deficient cells. (A-B) Representative images of primary basolateral dendrites and apical dendrite width in Thy1-YFP;CreER$^{+/+}$ x Dicer$^{f/f}$ cells with and without tamoxifen treatment. Scale bars = 30µm. (C) Reduction in the number of primary basolateral dendrites in tamoxifen treated pyramidal neurons of the CA1 compared to control cells, presented as mean ± SEM (n = 10 per group). (D) Quantitative analysis of mean ± SEM apical dendrite thickness in Thy1-YFP positive cells are presented. No significant difference in apical dendrite width between groups (n = 5 per condition). *$P < 0.05$, two-tailed t-test.



Figure

5. Increase in Thy1-YFP cell count. (A-B) Immunofluorescent visualization of Thy1-YFP cells in control animals and those with Dicer deleted. (C) Marked increase in the number of Thy1-YFP positive cells per hemisphere in the CA1 of immunolabeled sections of Dicer inactivated tissue (n = 5 per condition, 2 sections per animal). Data are presented as mean cell count ± SEM. *P < 0.05, two-tailed t-test, scale bar = 100 μm.

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